

SAS:dsh 12/4/06 4239-66898-01 619369 E-177-2000/2-US-02

PATENT
Attorney Reference Number 4239-66898-01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Court et al.

Application No. 10/692,553

Filed: October 23, 2003

Confirmation No. 1179

For: ENHANCED HOMOLOGOUS
RECOMBINATION MEDIATED BY
LAMBDA RECOMBINATION PROTEINS

Examiner: Jennifer Ann Dunston

Art Unit: 1636

Attorney Reference No. 4239-66898-01

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Attorney or Agent
for Applicant(s)

Date Mailed December 4, 2006

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TRANSMITTAL LETTER

Enclosed for filing in the application referenced above are the following:

- Letter Regarding Declaration of Dr. Ellis
- Declaration Under 37 C.F.R. § 1.131 (executed by Hillary M. Ellis)
- The Director is hereby authorized to charge any additional fees that may be required, or credit over-payment, to Deposit Account No. 02-4550. A copy of this sheet is enclosed.
- Please return the enclosed postcard to confirm that the items listed above have been received.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

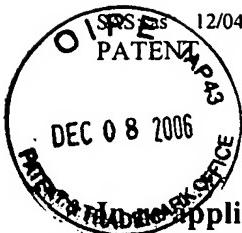
By

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cc: Docketing

TRANSMITTAL - Page 1 of 1



12/04/06 620080 E-177-2000/2-US-02

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LETTER REGARDING DECLARATION OF DR. ELLIS

On October 16, 2006, Applicants submitted a Petition to Accept a Declaration

Signed by Other Than All the Inventors. This petition requested that the U.S. Patent and Trademark Office (USPTO) accept a Declaration Under 37 C.F.R. § 1.131 that had not been executed by Hillary Ellis. On November 3, 2006, the undersigned was able to contact Dr. Ellis at a new address. Enclosed is a Declaration under 37 C.F.R. § 1.131 executed by Hillary M. Ellis on November 6, 2006. Applicants request that the USPTO accept this executed Declaration under 37 C.F.R. § 1.131. The Petition to Accept a Declaration Signed by Other Than All the Inventors is no longer required.

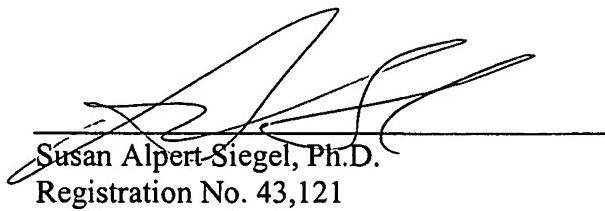
The Examiner is requested to contact the undersigned for a telephone interview if anything further is required to accept the Declaration under 37 C.F.R. § 1.131 executed by Hillary M. Ellis.

Respectfully submitted,

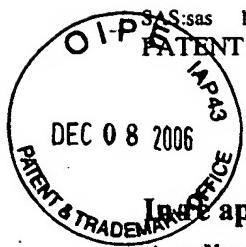
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By



Susan Alpert-Siegel, Ph.D.
Registration No. 43,121



SAS:as 11/03/06 555051 E-177-2000/2-US-02

Attorney Reference Number 4239-66898-01
Application Number 10/692,553

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor application of: Court et al.

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Examiner: Jennifer Ann Dunston

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12/4/06

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DECLARATION UNDER 37 C.F.R. § 1.131

We, Neal Copeland, Daiguan Yu, Hilary M. Ellis, Donald E. Court, E-Chiang Lee, Nancy A. Jenkins, and Pentao Liu, declare as follows:

1. We are the inventors of the above-identified application, which is a continuation of U.S. Patent Application No. 10/366,044, filed February 12, 2003, which is a continuation-in-part of PCT Application No. PCT US01/25507, filed August 14, 2001, which claims the benefit of U.S. Provisional Application No. 60/225, 164, filed August 14, 2000 and claims the benefit of U.S. Provisional Application No. 60/271,632, filed February 21, 2001.

2. It is our understanding that the claims 1, 3, 4 and 13 are rejected as allegedly being anticipated by Cassanova et al., Genesis 32(2): 158-160, published online February 13, 2002.

3. We conceived of, and reduced to practice, a method for generating a vector for conditional knockout of a gene in a cell including a de-repressible promoter operably linked to a

nucleic acid encoding Beta and Exo, as claimed in claims 1, and 2-13, prior to February 13, 2002, in the United States.

3. The methods of claims 1, 3, 4 and 13 were conceived of prior to February 13, 2003. Selection cassettes for use in the claimed methods were made and improved prior to February 13, 2002; some of the experimental work conducted prior to February 13, 2002 is described below. Photocopies of Dr. Liu's laboratory notebook pages, labeled pages 1-10 are submitted herewith. The photocopied pages are referred to below as "the laboratory notes." Dates on these pages have been redacted. Prior to February 13, 2002, we performed the following experiments in the United States, which are documented on the laboratory notebook pages:

We constructed a plasmid that including a selectable marker (specifically a kanamycin/neomycin resistance marker) flanked by a pair of recombining sites (specifically LoxP). This plasmid was designed to introduce the recombining site into a genomic locus on a bacterial artificial chromosome (BAC) or a plasmid. A diagram of this plasmid, and a restriction map of this plasmid is shown in the laboratory notes, see page 1. The selection marker is called PL400.

We also constructed PL428 and PL430 which were additional plasmids for introducing recombining sites (LoxP sites) into the 5' and 3' sides of a genomic fragment of the Ctip2 locus. This is documented in the attached photocopy of Dr. Liu's laboratory notes, labeled page 2. DNA fragments of PL428 and PL430 were restriction digested or amplified by polymerase chain reaction. These fragments, containing the selectable marker (Kan-Neo) flanked by two recombining sites (LoxP) and having homology arms, were electroporated into E. Coli cells containing a de-repressible promoter (pL) operably linked to a nucleic acid encoding Beta and Exo. The production of kanamycin resistant cells is documented at the bottom of page 2 ("Kan^R"). A recombinase (Cre) is used to excise the nucleic acid encoding the selectable marker to leave a single first recombining site in the gene, as indicated on the right side of page 3 of the laboratory notes.

To clone a mouse genomic fragment from a BAC using recombineering, in order to make the conditional targeting vector, a retrieval vector (PL433) was constructed. PL433

includes two short DNA fragments from the end of the genomic DNA fragments. There is a MC1TK (thymidine kinase, a second selectable marker) in the backbone of this plasmid, negative selection could be used in embryonic stem cells with this conditional targeting vector. The production of PL433 is documented on page 4 of the laboratory notes.

The PL433 plasmid was electroporated into E. coli cells wherein the de-repressible promoter was de-repressed. Two colonies were examined by digesting the DNA with restriction enzymes. The restriction pattern documented that the selectable marker (TK) was inserted flanked by a second pair of recombining sites (LoxP). This produced plasmid PL435, shown on page 5 of the laboratory notes, which contained the genomic fragment (Ctip2) for making the targeting vector.

The DNA insert (2.8 kb in length) from PL430, which contained the selection marker (Kan-Neo) flanked by two recombining sites (loxP) was co-electroporated into bacterial (E. Coli) cells including a derepressible promoter (pL) operably linked to Gam and Exo. The cells were heat induced to insert the first recombining site into the Ctip2 locus. The correctly targeted plasmid was re-transformed into bacterial cells (E. coli). The loxP-flanked Kan marker was excised in the E. coli to leave a single loxP site in the genomic DNA. (see page 6 of the laboratory notes, top panel). This new plasmid was co-electroporated with the DNA fragment from PL436 containing the Neo-Kan selection marker also flanked by a second pair of LoxP sites. This resulted in the production of plasmid PL437. PL437 is the conditional knock-out vector that will allow deletion of the last exon of Ctip2 (see page 6 of the laboratory notes, bottom panel). The configuration of PL437 as a conditional targeting vector was confirmed using restriction digestion, as shown on page 7 of the laboratory notes.

A vector for conditional knock-out of the Evi9 locus was generated. This conditional targeting vector was designed to delete exon 4 of the Evi9 gene. The construction of this vector is shown on page 8 of the laboratory notes.

PL438 was a plasmid that contained a first pair of recombining sites (two LoxP sites, also called "floxed") flanking a selection marker (Neo-Kan), and flanked by two PCR amplified genomic DNA fragments. These genomic fragments could be used as homology arms in recombineering. The insert from this plasmid placed the floxed selection marker (Kan) into the 5' side of exon 4 (within exon 3) of the Evi9 gene. This plasmid could be used to introduce the first recombining sites into a BAC.

PL440 was a plasmid also contained a pair of recombining sites (LoxP or “floxed”) flanking a selection marker (Neo-Kan) and flanked by two polymerase chain reaction (PCR) amplified genomic DNA fragments. PL440 was used for recombineering. The insert from PL440 was used to place a floxed selection marker (Kan) into the 3’ region of exon 4 (in intron 4) of the Evi9 gene. This plasmid could be used to introduce the second pair of recombining sites into a BAC.

PL441 was then constructed. This is a retrieval vector for retrieving the Evi9 genomic DNA fragment from an Evi9 BAC (see the bottom of page 8 of the laboratory notes). Linearized PL441 was electroporated into an Evi9 BAC (called “C3,” see page 9 of the laboratory notes). The retrieved plasmid was called PL442. PL442 was co-electroporated with the insert from PL438 to place a floxed Neo-Kan selectable marker into intron 3 of Evi9 (see page 9 of the laboratory notes).

The targeted plasmid was transformed into *E. coli* expressing a recombinase (“Cre”) to excise the selectable marker. This left a single LoxP site in intron 3 of Evi9. The production of this allele is shown in the top panel on page 10 of the laboratory notes.

The excised plasmid was then co-electroporated with the insert from PL440 to place a second floxed selectable marker (Neo-Kan) into intron 4 of Evi9. Thus, the plasmid PL443 was produced, which is a conditional targeting vector that could be used to delete exon 4 (located between intron 3 and intron 4) of Evi9. The production of PL443 is shown in the bottom panels on page 10 of the laboratory notes. We were aware that an Frt site could be used as a recombining site in the place of a loxP site, and that Flp could be used as the recombinase. A strain of *E. Coli*, EL250 was created that expresses Flp.

4. These results demonstrated: (1) homologous recombination could be used to insert a nucleic acid encoding a selectable marker (Neo-Kan) flanked by a pair of first recombining sites (LoxP) into a first site (one intron) in a gene (Evi9 or Ctip2) in vector including bacterial artificial chromosome (Evi9 or Ctip2), (2) homologous recombination could be used to insert a nucleic acid encoding a selectable marker (Neo-Kan) flanked by a pair of second recombining sites (LoxP) and a first recombining site into a second site (a second intron) in the gene (Evi9); (3) the nucleic acid encoding the selectable marker could be excised with a first recombinase specific (Cre) specific for the recombining sites, leaving a single first

recombinant site in the gene (Evi9 or Ctip2), and (4) the nucleic acid encoding a selectable marker (Kan-Neo) could be excised with a recombinase (Cre) specific for the second recombining sites. Two recombining sites remained in the gene following excision of the nucleic acid encoding the selectable marker, thus generating a vector for conditional knockout of the gene (Evi9 or Ctip2). *E. coli* strains were created that expressed Flp, so that FRT recombining was used. The homologous recombination was performed in bacterial cells including a de-repressible promoter (pL) operably linked to a nucleic acid encoding Beta and Exo.

5. All statements made herein and of our own knowledge are true and all statements made on information are believed to be true; and further, these statements were made with the knowledge that willful false statements and like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements made may jeopardize the validity of the application or any patent issuing thereon.

Date _____

Neal G. Copeland

Date _____

Daiguan Yu

Date November 6, 2006


Hilary M. Ellis

Date _____

Donald L. Court

Date _____

E-Chiang Lee

Date _____

Nancy A. Jenkins

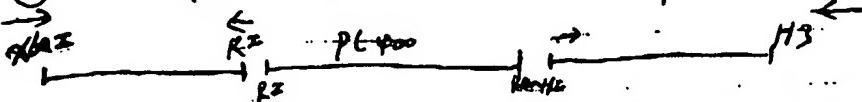
Date _____

Pentao Liu



PC fragments for making target vector to introduce 1st GxP.

(1) X6a2/R2 X6a2-10866 Ctip², R2-11274 Ctip²
 (2) Bant2-H3 Bant2-11275 Ctip², H3-11532 Ctip²



2λ	X6a2/R2	0
2λ	PL400	2
2λ	Bant2/H3	0
2λ	TSKf	2
1λ	10X6ff2	1
1λ	T4 Lyse	1



1 λ PVA
 1 λ DNP
 1 λ PVA
 1 λ P-2
 1 λ PVA

2 λ
 PL400

X6a2/X6a2
 X6a2/H3
 X6a2

Bant2/H3 X6a2



digest PL428 #16, PL430, #2 #4 w/ $\text{Nhe}^1/\text{Sal}^1/\text{Pvu}^1$
to excise the targeting cassettes.

15 λ plasmid

4 λ buffers

2 λ enzymes (Nhe^1 , Sal^1 , Pvu^1)

20 λ H₂O

4 λ 2 λ

37 $^\circ$ 1.5 hr. gel purify

open column gel purified

QIAquick

3 λ 20 μg A

use 3 λ + 1 λ PL424 (110 μg) ~~#27~~

42 $^\circ$ \rightarrow 240 Kan^r ←
32 $^\circ$ \rightarrow 4 electroformation. Time constant 46.4
9.0 min. or more if needed.

can also amplify the wild-targeting vector by PCR

① amplify PL428 w/ XbaI-13264-Cfip2

H3-13933-Cfip2

② PL430 w/ XbaI-12966-Cfip2

H3-1

use $\text{Nhe}^1/\text{Sal}^1/\text{Pvu}^1$
buf

PCR products were purified

by QIAquick \rightarrow 2 λ QEB

PL428 = 360 μg /L

PL430 = 330 μg /L

1 λ dNA
1 λ dNTP
1 λ primer
1 λ primers
H₂O

5 λ 0.95 μl TBS

use 1 λ PL424 #27 (110 μg)

plus 1 λ PL430 330 μg or 2 λ PL430 660 μg . If use 32 $^\circ$ (cells)
168 Kan^r 176 Kan^r 0 Kan^r

seems increased targeting DNA does not increase Kan^r
might need more target DNA (PL424 #27).

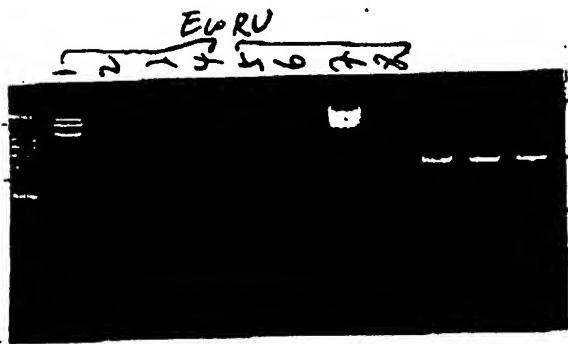
miniprep.

(2)

#1 → #4 from targety w/ purified plasmaed insert p(4)°.

#5 → #8 from ... w/ p/c of p(4)°.

~~plasmaed~~ ~~or~~ ~~target~~ insert from plasmaed is better.



use #2 electroop → E(350) → Kan⁺
#2 → C/R induced E(350) → Kan⁺ Amp/r
→ #1, #2 from
→ #3, #4 still mixed after popout



use #4 mixed-popout cells direct &
and electrooporated w/ pL428 purified cascade
4V_d induced. no Kan along...
indicating Co-electroporation is a must

primers

- ① NotI-ctfp2-ret-5'-1-4001
- H3-ctfp2-ret-5'-2-4371
- ② Hs-ctfp2-ret-3'-1-17779
- SpeI-ctfp2-ret-3'-2-18145

- ③ BamHI-CG9650-5'
- XbaI-CG9650-3'
- ④ Same as ③

- ⑤ BamH2-CG9650-3
- XbaI-CG9650-3'

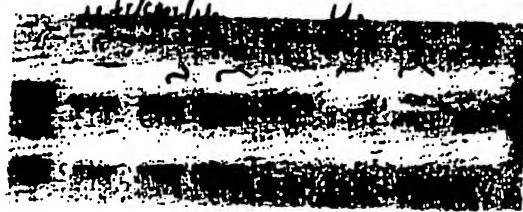
- ⑥ Same as ⑤

#1, #2 work
#3 → #6 do not work.

#1, #2 were purified as DNAse and digested
with appropriate enzymes, and again purified
by QIA.

11-04-01
#1 Cut w/ NotI/H3, #2 Cut w/ H3/SpeI, pL(25) NotI/SpeI
ligation.

3λ #1
3λ #2
2λ pL(25)
10x T4P
10x H2O
10x Taf
20x H2O



DNA

C5



Fly adult RT. Random priming

Fly adult RT. Oligo dT priming

Fly adult RT. Random priming

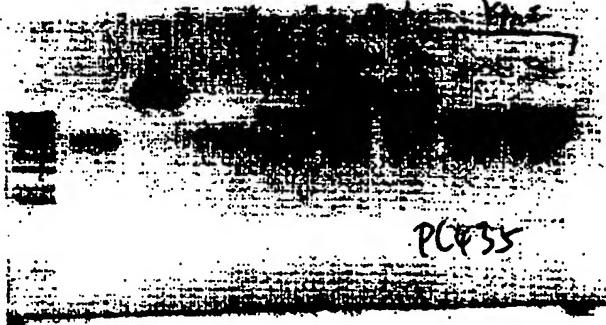
Fly adult RT. Oligo dT priming

+ λ DNA
+ λ dNTP
+ λ primer
+ λ primer
+ λ H2O
5λ 0.75λ 10x Taf
20λ H2O

pL433

refrigerator
PL 433 (1307/2) - 42°C
42°C induced ctg C5 BAC
amp plate

total colonies : 42°C $1200 \times 8 = 9600$
32°C $11 \times 8 = 88$



(K)

PL435 co-electroporation = PL435 insert to target the 1st LxP
 9AM incubate to file. Electroporated plasmid.
 EL350

42°C 100 colonies, 32% 10 colonies.

no mixture of targeted plasmid

pick up ~~two~~ four. 2 are correlated #3, #4.

#4 dilute 100x frozen

→ EL350 (core) to pop out

→ DH10B

Bands. 14.5 kb wt.
7 kb

KV 11 kb → 10 kb

9 kb



directly from pop out colonies. EL350 (pop out) → 100 colonies. EL350 (targeted) → 10 colonies. DH10B thoroughly read dilution. 9AM EL350 incubate two → 100 colonies 10 hrs → min prep

One has songta, and Hongta dark.

use the one are 1λ + 1λ PL436 cassette (150ng/λ)

↓
42°C induced EL350.

42°C 102 colonies

32°C 9 colonies. pick up 4 → 32°C → o/v



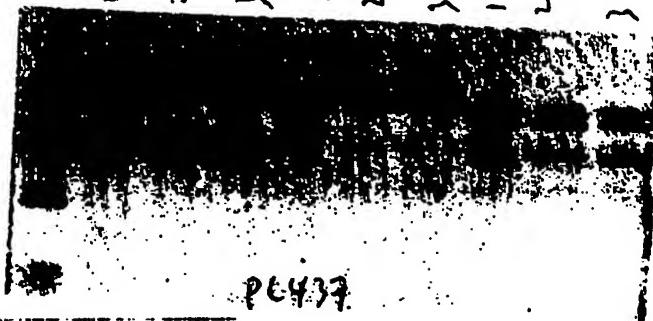
#L1, 3, 4
are mixed
with targeted
and non-targeted
take 1ng of #4
→ DH10B

PL437

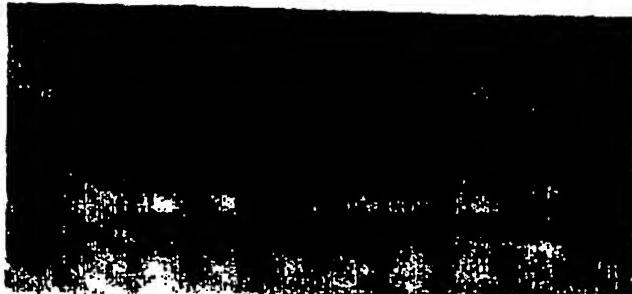
(6)

Wings? 3 colors of 2H108

Batt = RUV Xba =



of pl
Conditional
targetly
Vector



Evi9 exon 4 refrig. & 3' 6xP... P CR

- primers
- | | DNA | |
|--|----------------|------------|
| 1. Metz-Evi9-exon4-refrig 5'-1
H3 - Evi9-exon4-refrig 5'-2 | { BAc C3
A5 | 1λ DNA 4 |
| 2. H3 - Evi9-exon4-refrig 3'-1
H3 SPC-Evi9-exon4-refrig 3'-2 | { Same | 1λ NTP 4 |
| 3. XbaI-Evi9-exon4-3'-L1
RI - Evi9-exon4-3'-L2 | { .. | 1λ prim 1 |
| 4. BamHI-Evi9-exon4-3'-K1
H3 - Evi9-exon4-3'-R2 | { .. | 1λ prim 2 |
| | | 2λ RNA 84 |
| | | 5λ 2 λ |
| | | • 7λ 1 λ 3 |
| | | 20λ RNA |

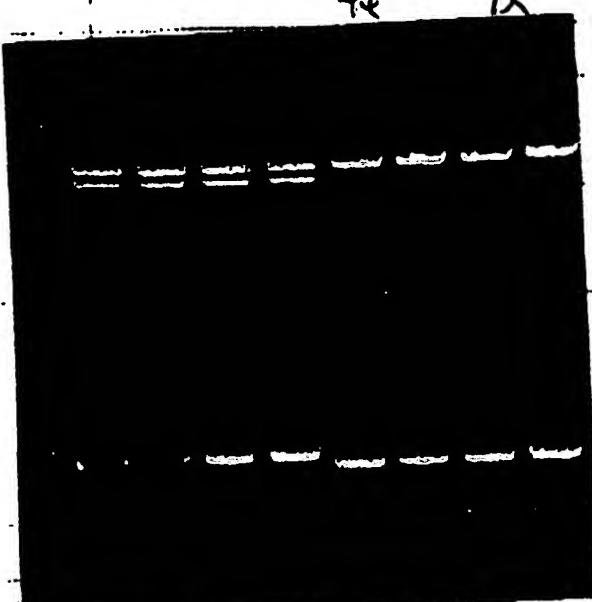
not purified w/ columns and cut. → purify

1. 3λ
2. 3λ
PCKS³ ATP/ATP 2λ
Eco RI 1λ
T4 1λ

3. 28λ
4. 28λ
PCKS³ ATP/ATP 3λ
PCKS³ ATP/H3 2λ
10λ T4 1λ
1λ T4 1λ

pL4460: 6xp to 3' of Evi9, exon 4

pL4441. refrig vector for exon 4, Evi9



w/ SAC A5 C3 electroporated to EL350 \approx 30-40% Gluc.
 electroporated H3 cat pL441 \rightarrow A5. C3 BACs
 thousands. ~~long~~ clones recovered
 pick 4 2 (#1, #2) from A5, 2 (#3, #4) from C3
 10 hours at 32°C in App. mini-prep
 only #4 (clone A3) is correct, indirectly A5 BAC may not have any
 take 2λ #4, \rightarrow 200 λ TE. take 1λ \rightarrow DH10B (2λ) in LB
 plate 100 λ , 10³ clones grown up. picks
 #1 - #2. original #4 prep
 #3 - #4. original #2 prep (in-correct).

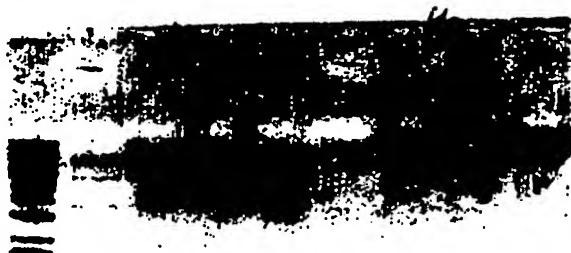


pL442 #1, $\xrightarrow{42^\circ\text{C}}$ long λ ... 3λ each electroporate \rightarrow EL350 FT^+

pL442 #2 \rightarrow long λ each

plate on wrong partitions (cm), re-dose

re-purified pL442 #1, long λ . use 2λ , use $75\mu\text{l}$ pL442 #1 \rightarrow EL350
 1 ml tube, 24 $^\circ\text{C}$ 20' static incubator (waitly for white both to turn) $\xrightarrow{42^\circ\text{C}}$ 50°C Kan. \rightarrow 12° Kan. pick up 4

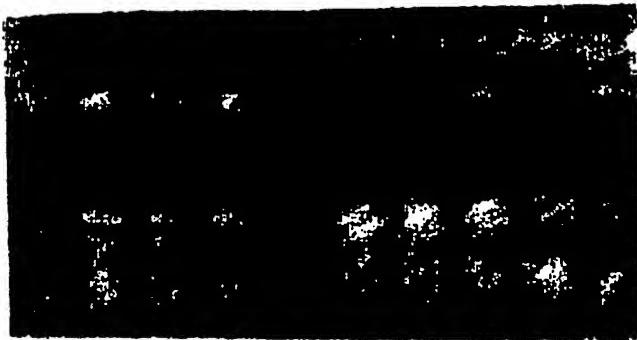


all are targeted.

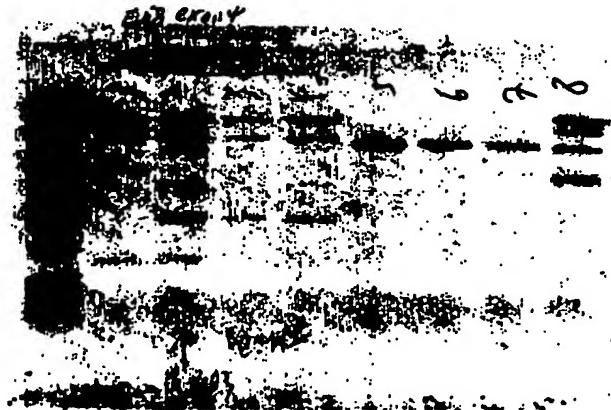
but #4 do not have WT contamination

\rightarrow used for plasmid

~~EVIG~~ evig exone conditional allele - Est CosP pop out.



1-1-02. targeting the pop out plasmid #2 w/ pL440 insect wks.
using the frozen El350 competent cells? $\approx 44 \text{ f.u.}^{200}$
 $2^{\text{nd}} \text{ pick up } P_{\text{GK}}$ pick up 4 colonies. 3 targeted. Transform #L \rightarrow DH10B kan^r plate,
pL443.



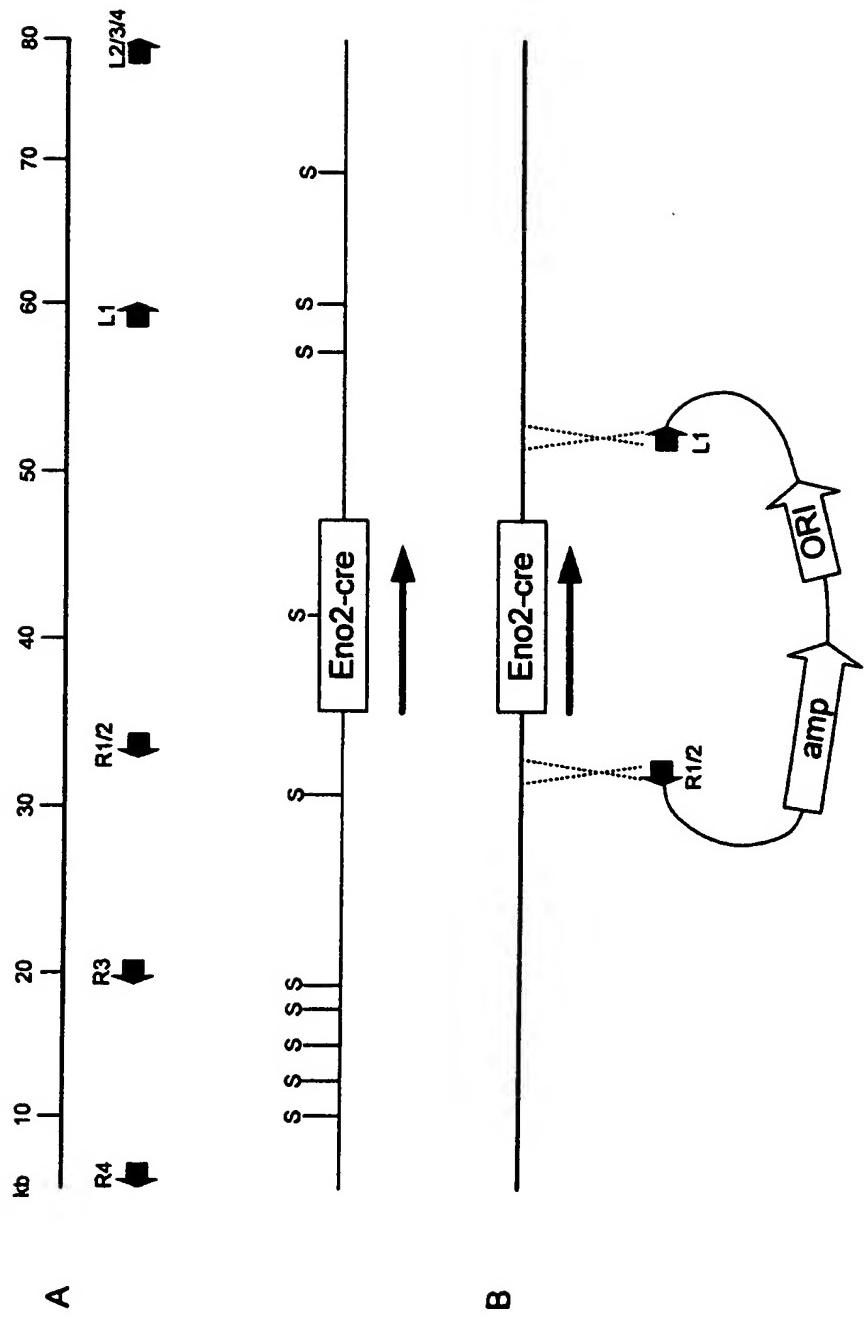


FIG. 8

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